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The effects of BDNF Knockdown on Neuroinflammation in the Male and Female rat brain

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ABSTRACT

Since Brain-Derived Neurotrophic Factor's (BDNF) discovery in 1982, we know that it plays a critical role in both cell survival and cell death within the central nervous system (CNS). It has been established that BDNF, serves as a regulatory body in the CNS, from development to adulthood by promoting neuronal health, survival, and maintenance. However, little is known about BDNF's role in the pathogenesis of neurological, neurodegenerative, and neuropsychiatric disorders. Therefore, in this study, we used western blots analysis, to examine the effects of global BDNF knockdown on the protein expression of multiple inflammasome and glial cell markers in healthy male and female rat brains. We hypothesize that BDNF heterozygous rats ($BDNF^{+/-}$ KD), will have altered protein expression of inflammatory markers due to BDNF loss, and that this may occur in a sex specific manner. To test this hypothesis, we examined the activation of the NLRP3 inflammasome pathway in male and female rat hippocampal tissue. When compared to wildtype (WT) controls, only $BDNF^{+/-}$ KD males showed a significant increased protein level expression of reactive oxygen species (TXNIP), and inflammasome marker Caspase-1. When taken together, all four inflammasome markers (NLRP3, ASC, Caspase-1, IL1-β) showed a visual pattern of increase only in the male hippocampi (HPC) and not in the females after $BDNF^{+/-}$ KD. In conclusion our results compel us to believe that BDNF reduction leads to priming of the inflammation in the brain and not a full-blown inflammatory state. This is observed in a sex specific manner, with effects seen only in the male HPC. These findings contribute information about BDNF's

involvement in neuroinflammation and will aid in the development of more effective treatment options for neurodegenerative, and neuropsychiatric disorders in the future.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE
OVERVIEW AND SPECIFIC AIMS

Statement of the Problem

In a healthy brain, BDNF serves as a regulatory body in the CNS from development to adulthood by promoting neuronal health, survival, and maintenance (Kazak & Yarim, 2017; Numakawa et al., 2010). Previous studies, conducted on neurodegenerative and neuropsychiatric disorders have indicated that, loss of BDNF protein expression leads disrupted BDNF down-stream signaling. It has been determined through prior research, deficits in down-stream synaptic signaling is the cause for many neurological disorders (Boger et al., 2011; Dansokho & Heneka, 2018; Lima Giacobbo et al., 2019; Pillai, 2008). A common symptom found among all these neurological disorders is BDNF reduction and chronic neuroinflammation (Andero, Choi, & Ressler, 2014; Balaratnasingam & Janca, 2012; Dansokho & Heneka, 2018; Mortezaee, Khanlarkhani, Beyer, & Zendedel, 2018; Russo-Neustadt, 2003). However, little is known about the molecular mechanisms of BDNF and its role in neuroinflammation (Lai et al., 2018; Lamkanfi & Dixit, 2012). In order to determine how BDNF loss affects neuroinflammation in the hippocampus we must look at it under non-pathologic conditions and if there are any sex differences. We also must examine what pathways that are affected specifically by neuroinflammation. In our study we hypothesize that BDNF heterozygous rats ($BDNF^{+/-}$ KD) will have altered protein levels of inflammatory markers due to reduction of BDNF expression, and that this may occur in a sex specific manner. We will test this hypothesis through examination of inflammation

within the Central Nervous System (CNS), specifically through examining the NLRP3 inflammasome pathway (NLRP3 pathway: ASC, Caspase1, and IL-1β).

Specific Aims

Aim 1: To understand the basal relationship between BDNF and how its neuronal functioning affects neuroinflammatory pathways. To do this, we knocked down BDNF in the rodent brain to determine if the reduction of BDNF induces activation of NLRP3 inflamamsome pathway proteins, namely, NLRP3, ASC, Caspase1, and IL-1β. This was followed by measuring upstream oxidative stress marker, TXNIP and overall inflammation by looking at glial cell activation.

Aim 2: To determine if sex differences play a role in BDNF regulation and neuroinflammation. We used sex-matched groups to compare post-mortem hippocampal tissue from male and female WT rat to $BDNF^{+/-}$ KD rats via western blot protein expression of reactive oxygen species (TXNIP), the NLRP3 inflammasome pathway (NLRP3, ASC, Caspase1, and IL-1β), and glial cell immune response (Iba1, and GFAP).

CHAPTER TWO
INTRODUCTION AND LITERATURE REVIEW

Brain Derived Neurotrophic Factor (BDNF): An Introduction to the Family of Neurotrophins

Sixty years ago the first neurotrophic factor was discovered, Nerve Growth Factor (NGF)(Levi-Montalcini, 1964; Mitre, Mariga, & Chao, 2017). NGF was found to be a secreted protein that was necessary for neuronal development and survival (Ceni, Unsain, Zeinieh, & Barker, 2014). Preliminary studies discovered that NGF receptors are expressed by peripheral neurons(Ceni et al., 2014; Dechant & Neumann, 2002). This discovery of NGF led to the identification of other neurotrophic factors. The second neurotrophin discovered was Brain derived neurotrophic factor (BDNF)(Russo-Neustadt, 2003). BDNF has been established as a major trophic factor within the central nervous system (CNS) (D. Y. Liu et al., 2015). BDNF impacts multiple facets of neuronal function, including growth, morphology, and synaptic plasticity (Gomez-Palacio-Schjetnan & Escobar, 2013). When BDNF is absent neuronal function and plasticity decrease, suggesting that BDNF integrates circuits and signaling pathways throughout the CNS (Dechant & Neumann, 2002; D. Y. Liu et al., 2015; Mitre et al., 2017).

Previous research has shown that neurotrophins have a regulatory role in the immune response within the CNS (Bothwell, 2016). Neurotrophins are protein growth factors, are necessary for neuronal survival, differentiation, and maintenance from development to adulthood (Bibel & Barde, 2000; Bothwell, 2014; Mitre et al., 2017). There are four major neurotrophins: NGF, BDNF, NT-3, NT-4; all four trophins impact

bidirectional signaling between the neurosensory network and immune cells (Barbacid, 1995; Bothwell, 2016; Manti, Brown, Perez, & Piedimonte, 2017).

What is unique about neurotrophins is that they are vital for CNS and PNS function in their immature form, referred to as a proneurotrophins, and their mature form (Gibon & Barker, 2017). Once the proneurotrophin is synthesized and is cleaved intercellularly as well as extracellularly it transforms into its mature state (Mitre et al., 2017; Rafieva & Gasanov, 2016). Both pro and mature neurotrophins have conflicting roles as they can promote cell survival but also cell death, which is unique in the CNS (Dechant & Neumann, 2002; Gibon & Barker, 2017; Twiss, Chang, & Schanen, 2006).

Neurotrophins provide support after any insult or injury within the CNS (Ceni et al., 2014; Rafieva & Gasanov, 2016). A commonality found among all neurotrophins is their ability to synthesis in cells as long precursors are present and contain a N-terminal signal peptide and can follow the N-terminal signal (Bothwell, 2016; Mitre et al., 2017; Rafieva & Gasanov, 2016). This is what has propelled us to further investigate BDNF's pathways and receptors in order to understand its cellular and molecular function within the CNS and what happens when it is reduced or absent (Ceni et al., 2014; Khan & Smith, 2015; Skaper, 2008a, 2008b, 2012). BDNF is known to have a high affinity for binding to neurotrophic tyrosine kinase (Trk) receptor, (Davies, 2008; Skaper, 2012; Teng, Felice, Kim, & Hempstead, 2010) it has a low affinity for binding to pan neurotrophin receptor (p75NTR) (Davies, Minichiello, & Klein, 1995; Patapoutian & Reichardt, 2001; F. Zhang, Kang, Li, Xiao, & Zhou, 2012). This review will be focusing on BDNF mechanisms,

function, receptors, neuro-pathobiology, clinical application and its role in CNS inflammation. (Khan & Smith, 2015).

BDNF Function

The function of BDNF has previously been identified as promoting neuronal survival, neuronal maintenance, and synaptic plasticity (Lima Giacobbo et al., 2019). As indicated in the previous section, and it is known that BDNF is a member of the neurotrophin family that serves as a regulatory body within the central nervous system from development to adulthood (Lima Giacobbo et al., 2019). What has not been mentioned is the cell biology of BDNF and what makes it a key regulator of cellular cognition processing resulting in complex behaviors (Aid, Kazantseva, Piirsoo, Palm, & Timmusk, 2007). When a BDNF knockout is created in a rodent model the animals only live a few weeks and exhibit sensory impairments (Aid et al., 2007; Barker, 2009; Begni, Riva, & Cattaneo, 2017) In our study we used a knockdown rodent model, to ensure that our animals reached adulthood and so we could study BDNF loss (Ernfors, Lee, & Jaenisch, 1994; Jones, Farinas, Backus, & Reichardt, 1994; Lima Giacobbo et al., 2019).

BDNF's gene expression in humans has a complex structure that incorporates multiple promoters, splice sites, and three UTR poly-adenylation sites, which results in a sophisticated transcript regulation of messenger RNA (Aid et al., 2007; Barde, Edgar, & Thoenen, 1982; Begni et al., 2017; Boulle et al., 2012; Martinez-Levy & Cruz-Fuentes, 2014). The cellular role of the RNA transcripts plays an important role in the epigenetic modification of the regulation of the BDNF gene transcription (Aid et al., 2007; Barker,

2009; Lima Giacobbo et al., 2019; Marini et al., 2004; Pruunsild, Kazantseva, Aid, Palm, & Timmusk, 2007).

To fully understand the function of BDNF, the gene's composition and expression must be understood (Boulle et al., 2012; Marini et al., 2004). The BDNF gene is composed of a common 3′-exon which encodes the immature form of BDNF called pro-BDNF protein (Martinez-Levy & Cruz-Fuentes, 2014), and is followed by multiple species dependent 5′-noncoding, promoter-regulated regions, and ending in a coding 5′-noncoding (Aid et al., 2007; Barker, 2009; Begni et al., 2017; Lima Giacobbo et al., 2019; Pruunsild et al., 2007). The gene's expression is regulated by a wide range of endogenous and exogenous stimuli, including chronic stress (Lupien, McEwen, Gunnar, & Heim, 2009; McEwen, 2012). The BDNF gene transcribes pro-BDNF, which is then cleaved into BDNF in the cytoplasm by the extracellular matrix or the endoprotease (Barker, 2009; Lima Giacobbo et al., 2019; Lu, Pang, & Woo, 2005).

BDNF acts on two independent receptors within the brain responsible for physiological brain functions (Borodinova & Salozhin, 2016). A complex system of downstream signaling cascades effecting both pro and mature BDNF through two major receptors: tropomyosin-related kinase B (TrkB) and pan 75 neurotrophin receptor (p75NTR) (Borodinova & Salozhin, 2016; Lu et al., 2005; Minichiello, 2009). BDNF and pro-BDNF will bind with a low affinity to p75NTR. When BDNF binds to p75NTR it starts a cascade activation of apoptosis (the cell death cascade) within the CNS (Bernard-Gauthier, Boudjemeline, Rosa-Neto, Thiel, & Schirrmacher, 2013). However, when mature BDNF binds to its high affinity receptor TrkB, numerous intracellular signaling

pathways become active including protein kinases pathways (MAPK, ERK, PI3K) pathways (Bernard-Gauthier et al., 2013; Lu et al., 2005; Minichiello, 2009). BDNF/TrkB-stimulated intracellular signaling is a major component of synaptic plasticity, neuronal survival and morphogenesis (Numakawa, Suzuki et al. 2010(Lima Giacobbo et al., 2019)). Additional studies, (including this study) are now being conducted to determine how deficiencies in BDNF contribute to the pathogenesis of several neurological diseases: sleep disorders, psychiatric disorders, Huntington Disease, Parkinson's disease, and Alzheimer's disease (Lima Giacobbo et al., 2019; Lu, Nagappan, Guan, Nathan, & Wren, 2013).

BDNF Signal Transduction

BDNF's directly impacts transcription, processing, and secretion are regulated by synaptic activity (Leal, Bramham, & Duarte, 2017). Since, BDNF's major role in the CNS is to sustain an enhancement of activity dependent synaptic activity within in the hippocampus promoting learning and memory (Kojima & Mizui, 2017; Kowianski et al., 2018)..Both experimental and clinical trials have shown that stress and depression decrease BDNF expression throughout the brain (Aid et al., 2007; Kozisek, Middlemas, & Bylund, 2008; Lupien et al., 2009; McEwen, 2012). The decrease in BDNF expression reduces neurogenesis; however, treatment with an antidepressant aim to reverse the effects of reduced BDNF expression (Kozisek et al., 2008; McEwen, 2012). Research suggests that stress both in childhood and adulthood can lead to structural changes in the brain. These structural changes result in the impairment of memory formation by blocking information acquisition in the neuronal networks that modulate mood (Kozisek

et al., 2008; Lima Giacobbo et al., 2019; Lupien et al., 2009; McEwen, 2012; Minichiello, 2009).

BDNF is a regulator of synaptic transmission, which is a key component of LTP in the hippocampus. The hippocampus is the brain region that is an integral part of the memory formation loop (Leal et al., 2017). Thus, understanding the effect BDNF has on LTP is necessary and in order to study BDNF the TrkB receptor, which is the receptor BDNF binds to, needs to be understood (Lu et al., 2005; Minichiello, 2009). BDNF's role in LTP has been most extensively studied in the hippocampus where it acts on both the pre and post synapse(Leal, Comprido, & Duarte, 2014). BDNF regulates mRNA transportation along the dendrites by acting during the translation phase within the synapse (Leal et al., 2014). BDNF does this through modulating the beginning of the elongation phase during protein synthesis by acting on specific microRNA (miRNA) (Leal et al., 2014). The transcription regulation that BDNF affects is believed to be one of the mechanisms involved in both short and long-term regulation of the synaptic proteome and how it effects synaptic plasticity (Leal, Afonso, Salazar, & Duarte, 2015; Leal et al., 2017; Leal et al., 2014; Lu, Nagappan, & Lu, 2014).The molecular mechanisms that BDNF activates triggering LTP are the TrkB receptor, rescue of L-LTP when protein synthesis is blocked, BDFN/TrkB regulation of dendritic spines (Nibuya, Morinobu, & Duman, 1995). These three mechanisms affect different forms of LTP in the hippocampus (Panja and Bramham 2014).

Neuropathology and BDNF

Neurodegenerative Disorders and BDNF

Everyday there are estimates of hundreds of millions of people around the world that are diagnosed with a neurological disorder. It is estimated that 47.5 million people worldwide are affected by dementia with the commonality of Alzheimer's Disease being the cause. The stark reality is that approximately 7.7 million new cases each year emerge with a neurodegenerative disorder's diagnosis (WHO, 2016; [http://www.who.int/features/qa/55/en/]) (Tejeda & Diaz-Guerra, 2017). Despite much research the etiology of these diseases, there is still so much that researchers do not know. The most glaring problem is understanding the disease's onset and progression (Lima Giacobbo et al., 2019). Similar to neurological disorders, neurodegenerative disorders also have been reported to have low BDNF levels followed by chronic neuroinflammation (ex: Alzheimer's Disease, Huntington's Disease, and Parkinson's Disease is neuroinflammation) (Hashimoto et al., 2005; Lu et al., 2013; Yuan et al., 2010) The molecular mechanisms of how BDNF at an excitatory synapse activates TrkB receptors effecting downstream signaling pathways (Hempstead, 2015; Leal et al., 2017). Our current treatment plans for these disorders rely on restricting the aversive symptoms, which is not curative (Michalski, Corrada, Kawas, & Fahnestock, 2015; Qin et al., 2017). While, cognitive testing and neuroimaging have provided doctors and researchers greater understanding of the progression of the diseases, they are still not able to detect the neurodegeneration in its earliest stages (Agrawal & Biswas, 2015; L. L. Wu, Fan, Li, Li, & Zhou, 2010; Zuccato & Cattaneo, 2014). Only after a catastrophic cascade of damage

has taken place are doctors able to resolutely diagnosis the disease(Fenner, Achim, & Fenner, 2014; Parain et al., 1999; Porritt, Batchelor, & Howells, 2005; Tejeda & Diaz-Guerra, 2017). Since the discovery of biomarkers, there has been a push to use them to aid in the early stage diagnosis of neurodegenerative disorders (Agrawal & Biswas, 2015; Jove, Portero-Otin, Naudi, Ferrer, & Pamplona, 2014; L. L. Wu et al., 2010; Zuccato & Cattaneo, 2014). BDNF is being considered as a biomarker for early detection diagnosis (Agrawal & Biswas, 2015; Barthel, Schroeter, Hoffmann, & Sabri, 2015; Jove et al., 2014; Lima Giacobbo et al., 2019; Parnetti et al., 2013; Zhu, Ploessl, & Kung, 2014).

A majority of neurological disorders display either BDNF dysregulation or loss of BDNF leading to impairment of the BDNF signaling pathway (Tejeda & Diaz-Guerra, 2017). BDNF/TrkB signaling in cell function plays a key role in the stability of the central nervous system, and subtle changes in expression or downstream signaling can have dire consequences such as neurological and psychiatric disorders (Michalski et al., 2015). In Alzheimer's Disease (AD) neurotrophic signaling is impaired, and excitotoxicity results in neuronal death, this patterns found in multiple progressive neurodegenerative disorders (Qin et al., 2017; Tejeda & Diaz-Guerra, 2017)..

Patients with AD have neurotrophic signaling impairment, excitotoxicity results in neuronal death, and this pattern is found in multiple progressive neurodegenerative disorders (Qin et al., 2017; Selkoe, 2002). Individuals with AD have a progressive decrease of synapses and neurons within the hippocampus and cortex, resulting in short term memory loss (Braak & Braak, 1991; Masliah et al., 1994). AD is identified by the development of senile plaques, which are comprised of extracellular deposits of misfolded

amyloid β-peptide (Aβ) and intracellular neurofibrillary tangles (Elliott, Atlas, Lange, & Ginzburg, 2005; Peng, Wuu, Mufson, & Fahnestock, 2005). BDNF levels are reduced in individuals with AD when compared to healthy patients which is believed to be a crucial factor in cognitive decline since mature BDNF promotes neuronal function (Michalski et al., 2015; Qin et al., 2017).

Huntington's Disease (HD) is an autosomal dominant neurodegenerative disorder initiated by CAG expansion of the huntingtin (Htt) gene, creating a faulty elongation of the polyglutamine (polyQ) tract at the Htt N-terminus (Tejeda & Diaz-Guerra, 2017; Zuccato & Cattaneo, 2014). This dysfunction or cell death of medium sized spiny neurons (MSNs) located within the striatum is the main symptom of disease onset. Through the progression of HD, MSNs are monitored (Zuccato & Cattaneo, 2014). MSN declines are characterized by motor, cognitive decline, and psychiatric decline (Ferrer, Goutan, Marin, Rey, & Ribalta, 2000). Like with AD individuals diagnosed with HD often have BDNF loss in their symptomatic stages (Ferrer et al., 2000). Notably, it has been indicated that a reduction of BDNF/TrkB signaling might be the cause for the susceptibility of MSNs in HD patients (Tejeda & Diaz-Guerra, 2017; Zuccato & Cattaneo, 2014; Zuccato et al., 2001). HD patients, additionally, have global reduction of neurotrophin expression throughout the brain that inhibits corticostriatal transportation (Ferrer et al., 2000; Gauthier et al., 2004; Zuccato & Cattaneo, 2014; Zuccato et al., 2001). This presents a unique problem within the CNS since a majority of BDNF within the striatum is synthesized and delivered anterogradely via cells from the cerebral cortex (Altar & DiStefano, 1998). Bizarrely, wild type Htt is a component of the motor complex that moves BDNF vesicles down

microtubules of the cell (Gauthier et al., 2004). When the polyQ tract becomes enlarged its causes the mutant Htt gene to overwhelm the complex, inhibiting the vesicle's movement creating a strengthen bond to Htt-associated protein 1 resulting in disease onset ((Altar & DiStefano, 1998)Gauthier et al., 2004). The disease onset if followed by a reduced expression of pro-BDNF, inhibiting proBDNF's apoptotic function (L. L. Wu et al., 2010; Zuccato & Cattaneo, 2014). When all these events co-occur, individuals with HD have a deficit in their ability to enlist motor proteins to microtubules, drastically reducing neurotrophin release and transportation(Dompierre et al., 2007; Engelender et al., 1997; Ferrer et al., 2000). Individuals with HD have a reduction of neurotrophic support and receptor availability disrupting the balance of Trkb signaling in the Striatum leading to cognitive and motor movement deficits (106-110)(Tejeda & Diaz-Guerra, 2017; Zuccato & Cattaneo, 2014; Zuccato et al., 2001).

Parkinson's Disease (PD) is one of the most prevalent movement disorders (Boger et al., 2011), its onset presents with a reduction/loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), and inhibited intracellular gathering of α-synuclein inclusions (also referred to as (Lewy bodies & Lewy neurites) (Parain et al., 1999; Porritt et al., 2005; Tejeda & Diaz-Guerra, 2017). Through postmortem analysis, individuals suffering from PD show a reduction of both BDNF mRNA and protein in the SNpc and Striatum (Benisty, Boissiere, Faucheux, Agid, & Hirsch, 1998; Howells et al., 2000; Miller et al., 2006; Parain et al., 1999; Porritt et al., 2005). When further analyzed at a structural and protein level, the changes found in the surviving neurons located in SNpc are small in PD patients (Mohammadi, Amooeian, & Rashidi, 2018; Tejeda & Diaz-

Guerra, 2017). However, this small protein change leads to an impairment of neurotrophin signaling to the TrkB receptor (Fenner et al., 2014; Tejeda & Diaz-Guerra, 2017). The subcellular distribution of TrkB-FL and TrkB-T1 in the disease progression of PD reduces catalytic receptor isoform within the dendrites inhibiting synaptic function (Ding et al., 2011; Hung & Lee, 1996). BDNF/TrkB signaling loss in the substantia nigra (SN) is particularly devastating because it increases the risk of dopaminergic cytotoxicity is believed to contribute to the onset and progression of PD (Benisty et al., 1998; Ding et al., 2011; von Bohlen und Halbach, Minichiello, & Unsicker, 2005). When examined in animal models reduction or loss of BDNF expression or downstream signaling through the TrkB receptor has shown motor dysfunction and loss in aged animals (Boger et al., 2011). Indicating that, BDNF loss can cause dopaminergic neuron loss in the SN in aged animals (Baydyuk, Nguyen, & Xu, 2011; Boger et al., 2011; Porritt et al., 2005; Tejeda & Diaz-Guerra, 2017; von Bohlen und Halbach et al., 2005).

Neuropsychiatric Disorders and BDNF

The pathophysiology of multiple psychiatric disorders appears linked to impairment of BDNF/TrkB signaling and expression (Tejeda & Diaz-Guerra, 2017). One of the most studied psychiatric disorders is depression (Benatti et al., 2016). Depression in the brain is the inability to employ adaptive plasticity through neurotrophic signaling in the hippocampus, and cerebral cortex (Nestler et al., 2002). Numerous studies have supported the claim that neurotrophins play a critical role in depression (Benatti et al., 2016; Krishnan & Nestler, 2010; Nestler et al., 2002). Patients who seek antidepressant treatment for chronic depression have increased BDNF in their blood posttreatment

(Brunoni, Lopes, & Fregni, 2008; Sen, Duman, & Sanacora, 2008). The mechanism of many antidepressants is it to increase the expression of TrkB mRNA inducing a state of rapid activation for the TrkB receptor (Nibuya et al., 1995; Rantamaki et al., 2007; J. C. Zhang, Yao, & Hashimoto, 2016). This rapid activation or the TrkB receptor plays a critical role in the efficacy of antidepressants that rely on the expression and signaling of the BDNF/TrkB pathway (Duman & Monteggia, 2006; Nestler et al., 2002; Rantamaki et al., 2007; J. C. Zhang et al., 2016). When BDNF is used as an antidepressant treatment, it has been reported to have the same therapeutic effects as antidepressants in animal models (Siuciak, Lewis, Wiegand, & Lindsay, 1997).

Atypical neurotrophin expression is a major symptom in the pathogenesis of Schizophrenia. Individuals with schizophrenia suffer from cognition and perception impairments (Tejeda & Diaz-Guerra, 2017), and have decreases in dendritic spines and synaptic plasticity (Chen et al., 2009; Glausier & Lewis, 2013; Siuciak et al., 1997). Multiple studies have demonstrated that patients with schizophrenia have reduced levels of BDNF(Chen et al., 2009). Additionally, patients with schizophrenia have changes in the BDNF protein and total mRNA located within the prefrontal cortex (Durany et al., 2001; Hashimoto et al., 2005; Weickert et al., 2003). BDNF regulation maybe altered through irregular DNA methylation, specifically at the growth arrest and DNA-damage-inducible β protein (GADD45b) (Gavin et al., 2012). GADD45b is a promoter that is diminished in patients with schizophrenia(Gavin et al., 2012). When patients have reduced levels of GADD45b and decreased TrkB mRNA and BDNF protein expression, the downstream signaling is severely diminished in the prefrontal cortex (Emamian, Hall, Birnbaum,

Karayiorgou, & Gogos, 2004; Hashimoto et al., 2005; Weickert et al., 2003; Yuan et al., 2010).

Epilepsy is viewed as steaming from abnormal neuronal excitability, resulting from exorbitant activation of TrkB-FL (G. Liu et al., 2013). For epilepsy to manifest, atypical TrkB signaling must occur through the PLCγ pathway (Gu et al., 2015). Because epilepsy is characterized by recurrent seizures, it can cause molecular changes in neuron and glial cell expression of the glutamate receptors and uptake transporters causing excitotoxicity, resulting in permanent brain damage (Barker-Haliski & White, 2015; Tejeda & Diaz-Guerra, 2017). In Vitro models of epilepsy illustrated that when BDNF expression is not appropriately regulated within the CNS, all downstream signaling is interrupted and negatively impacted (Danelon, Montroull, Unsain, Barker, & Masco, 2016; Xie et al., 2014). As indicated above, many neuropsychiatric disorders are caused by an abnormal regulation of BDNF/TrkB signaling. Ironically if this abnormality is corrected, BDNF could be consider to be a therapeutic treatment (Tejeda & Diaz-Guerra, 2017). More research is needed to examine the possibilities of restoring neurotrophic signaling (Tejeda & Diaz-Guerra, 2017). Restoration of just the BDNF/TrkB signaling pathway could lead to an increase in adult neurogenesis, increase synaptic plasticity, and serve as neuroprotection (Lu et al., 2013; Xie et al., 2014).

Stroke and BDNF

Stroke is one of the top five deadliest diseases in the United States, caused by a lack of blood flow to the brain usually caused by a blood clot or burst blood vessel, resulting in oxygen deprivation to the brain causing tissue damage (Guzik & Bushnell,

2017). Post, stroke recovery is a challenging road for many patients whom have lost motor function and is considered a long-term disability(Jorgensen et al., 1995). For stroke patients to regain motor function and motor skills neuroplasticity is required (Mang, Campbell, Ross, & Boyd, 2013). Current research is focused on promoting neuroplasticity in poststroke patients by optimizing BDNF expression (Mang et al., 2013).

As a result of lack of pharmacologic interventions and treatments for patients recovering from stroke, there is a continuous search into discovering novel treatment options for the long-term disability associated with stroke recovery (Berretta, Tzeng, & Clarkson, 2014). It is known that BDNF and its TrkB receptor are synthesized and expressed throughout the brain regulating neuronal activity. Recently, BDNF has been shown to be neuroprotective and aid in recovery of motor skills/function post-stroke(Berretta et al., 2014).

BDNF promotes neuroplasticity, neurogenesis, and is involved in neuroprotection (Mang et al., 2013). BDNF is essential for stroke victims to relearn and regain motor control throughout their recovery (Mang et al., 2013). Current stroke rehabilitation utilizes aerobic exercise to upregulate BDNF expression throughout the central nervous system (Cotman, Berchtold, & Christie, 2007; Kluding, Tseng, & Billinger, 2011; Kramer, Erickson, & Colcombe, 2006; Lambourne & Tomporowski, 2010; Rand, Eng, Liu-Ambrose, & Tawashy, 2010). Aerobic exercise would naturally increase BDNF expression and aid motor learning through promoting neuroplasticity in stroke victims (Cotman & Berchtold, 2002; Cotman et al., 2007; Knaepen, Goekint, Heyman, & Meeusen, 2010). However, aerobic exercise alone does not promote neuroplasticity. Exercise creates a

conducive environment that will support neuroplasticity, which in many cases is the first step towards recovery and motor rehabilitation (Kleim, Cooper, & VandenBerg, 2002; Mang et al., 2013).

Traumatic Brain Injury and BDNF

Traumatic Brain Injury (TBI) has become a worldwide cause of death/disability for teenagers, adults, and the elderly (Bruns & Hauser, 2003; da Silva Meirelles, Simon, & Regner, 2017; Fleminger & Ponsford, 2005; Leibson et al., 2011). It is estimated that around 10 million people worldwide are annually affected by TBIs (Chauhan, 2014). Patients suffering from a severe TBI have a 50% mortality rate that is unfortunately due to the lack or restorative treatment options (Hyder, Wunderlich, Puvanachandra, Gururaj, & Kobusingye, 2007; McDonald, Sun, Agoston, & Shultz, 2016). A majority of TBIs are associated with a loss of homeostasis within the CNS caused by external trauma (Bruns & Hauser, 2003; Fleminger & Ponsford, 2005). External trauma in this instance is any insult or injury to the brain resulting in damage of brain tissue (McKee & Daneshvar, 2015; Thakkar et al., 2016).

TBIs occur in two phases. The primary phase is the external insult and is identified with temporary symptoms: damage to vasculature, axons, neurons, and glial cells (Stoica & Faden, 2010; Wurzelmann, Romeika, & Sun, 2017). The secondary phase also referred to as the second injury is after the immediate death of cells, causing a cascade of biochemical changes around the primary injury (Stoica & Faden, 2010; Wurzelmann et al., 2017). The biochemical cascade leads to further loss of neural tissue and function (Stoica & Faden, 2010; Wurzelmann et al., 2017). Unfortunately, there is no

pharmacologic intervention that will restore or rescue damaged neural tissue. A majority of treatment options for TBI are symptomatic focused, with the goal of either neurodegeneration or neuroprotection (Wurzelmann et al., 2017).

To determine the severity of the TBI in patients, clinicians use different metrics. One of the most widely used scales for diagnosis is the three-tiered classification system called the Glasgow Coma Scale (GSC). The GSC allows clinicians to rate the injury as mild, moderate, or severe (Chieregato et al., 2010). TBI recovery today is dependent upon multiple factors: age, GCS score, and pupillary response (McDonald et al., 2016).

BDNF's role before a TBI is to promotes cell survival, cell growth, plasticity, and long-term memory (Lorente, 2017). Out of all four neurotrophins BDNF is expressed more than the others in humans (McAllister, Katz, & Lo, 1999). Studies on BDNF expression post-TBI have delineated that the protein has a role in the pathophysiology of trauma, but its exact role is disputed. There are studies that have indicated that BDNF is neuroprotective (Kim & Zhao, 2005; Oyesiku et al., 1999; H. Wu et al., 2008) and other studies that report it is neurodegenerative (Felderhoff-Mueser et al., 2002; Shetty, Rao, Hattiangady, Zaman, & Shetty, 2004). Clinical trials also reported contradictory results; some studies reported that patients had higher levels of BDNF post-TBI than the controls while others reported lower levels of BDNF post TBI (Failla, Conley, & Wagner, 2016; Korley et al., 2016). These conflicting results could be because a majority of clinical studies did not determine if they were measuring the immature proBDNF form or the Mature BDNF form (Koshimizu, Hazama, Hara, Ogura, & Kojima, 2010). BDNF in its mature form is neuroprotective while proBDNF is neurodegerenative (da Silva Meirelles

et al., 2017). Further research into the crucial role BDNF plays for the brain's homeostasis is needed in order to increase its efficacy as a possible treatment option for post TBI recovery (da Silva Meirelles et al., 2017).

Neuroinflammation

The Role of BDNF and Neuroinflammation

BDNF is one of the most extensively studied neurotrophins in both healthy and disease models due to its pathophysiology that is ubiquitous with numerous neurological disorders (Lima Giacobbo et al., 2019). With the rise of neurological disorders worldwide, there is a need for the development of more novel treatments as well as a further understanding of the etiology of neurologic disorders (Whiteford et al., 2013). Since numerous neurological disorders are caused by environmental factors it is important to study outside factors as well (Lupien et al., 2009; Whiteford et al., 2013). Stress, for example, is an environmental factor that is well known to initiate a cascade of health problem. Stress is associated with stroke, heart attack, cognitive impairment, metabolomic, mood, and endocrine changes (Lupien et al., 2009). Stress can wreak havoc on the body and the brain, and chronic stress can activate pro-inflammatory glial cells in the central nervous system that creates a pro-inflammatory environment within the brain (McEwen, 2012). To combat the pro-inflammatory environment, anti-inflammatory cytokines, glial cells, and neurotrophic factors are released to promote neuronal survival within the brain (Thakkar et al., 2016). Subsequently, neurotrophic

factors are known to promote neuronal survival (Lima Giacobbo et al., 2019; McEwen, 2012).

After any injury or insult in the central nervous system, inflammatory signals are triggered causing multiple cascades signals throughout the cell leading to structural and molecular changes (Lima Giacobbo et al., 2019). Two types of neuroinflammation occur within the brain: pro-inflammatory and anti-inflammatory, and unfortunately both can result in apoptosis (cell death) (Marini et al., 2004). Glial cells act as the brain's immune system response, particularly microglia and astrocytes that play a key role in the pathological onset of the inflammatory process. Microglia release pro-inflammatory cytokines, increasing the chances for neurotoxicity (Marini et al., 2004). As long as the inflammatory signal is present, it can cause numerous neurological consequences such as cognitive impairment and neurological and psychiatric disorders (Lima Giacobbo et al., 2019; Marini et al., 2004; Oeckinghaus & Ghosh, 2009; Tilstra, Clauson, Niedernhofer, & Robbins, 2011).

Thus, neurological disorders that have down regulation of BDNF release or loss, in general, have caused many researchers to search for a link between neuroinflammation and BDNF expression in the central nervous system (Lai et al., 2018; Lamkanfi & Dixit, 2012; C. L. Wu, Hwang, Chen, Yin, & Yang, 2010). Since neuroinflammation is a key part of onset/progression of a neurological disorder, our study aims to add information on what happens when BDNF loss in a naïve animal occurs. Will the BDNF loss prime the brain for neuroinflammation? If BDNF loss primes the activation of oxidative stress (TXNIP), the NLRP3 pathway will activate, resulting in a

neuroinflammatory response inside the cells within the brain (Lima Giacobbo et al., 2019; Mangan et al., 2018; Xu et al., 2018).

NLRP3 Inflammasome Pathway

The inflammasome was first discovered in 2002 by Dr. Jurg Tschopp at the university of Lausanne (Martinon, Burns, & Tschopp, 2002). The discovery of the inflammasome revealed that inflammasomes are multiprotein complexes, they have cytoplasmic localizations, and when activated form the inflammasome complex (Guarda et al., 2011; Petrilli et al., 2007). When activation of the NLRP3 pathway occurs when the cell receives a distress signal triggering the oligomerization of the inflammasome pathway (Jo, Kim, Shin, & Sasakawa, 2016; Martinon et al., 2002). Once the inflammasome pathway is constructed it releases cytokines, resulting in a proinflammatory response within the brain (Petrilli et al., 2007).

When inflammation occurs in the brain it sends out signals that trigger the cytosolic innate immune signaling receptor NLRP3 (Mangan et al., 2018). Once triggered, a cascade of activation happens within the NLRP3 pathway (Hornung & Latz, 2010; Lamkanfi, 2011). Following activation, recruitment of the adapter apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) occurs (Davis, Wen, & Ting, 2011; De Nardo & Latz, 2011; Jo et al., 2016). When ASC is activated it causes another chain link reaction by causing pro-caspase-1 to cleave into its active form Caspase-1 (Jo et al., 2016). Caspase 1 then leads to the maturation of two proinflammatory interleukin (IL)-1 family cytokines: IL-1β and IL-18 (Davis et al., 2011; De Nardo & Latz, 2011; Lamkanfi, 2011; Martinon et al., 2002). The result is an inflammatory

response with the release of active cytokines in the brain causing pyroptosis (De Nardo & Latz, 2011; Jo et al., 2016; Lamkanfi, 2011).

Research on the molecular mechanisms involved in the activation of the NLRP3 pathway reveals believe that it occurs in two ways: transcription and post-translation(Jo et al., 2016). To fully initiate the NLRP3 inflammasome pathway, a multiple protein complex must be assembled through the binding of ASC, and pro-caspase-1 resuls in an active inflammatory complex (Perregaux & Gabel, 1994; Petrilli et al., 2007). Only a few molecule mechanisms have been proposed to induce the activation of the NLRP3 pathway: potassium efflux, lysosomal destabilization/rupture, and mitochondrial reactive oxygen species (ROS) generation (Gurung, Lukens, & Kanneganti, 2015; Heid et al., 2013; Jo et al., 2016; Okada, Matsuzawa, Yoshimura, & Ichijo, 2014; Perregaux & Gabel, 1994; Petrilli et al., 2007). Studies have shown that anomalous activation of the NLRP3 inflammasome pathway is connected to the pathogenesis of inflammation in neurodegenerative disorders, psychiatric disorders, and stroke (De Nardo & Latz, 2011; Lamkanfi, 2011). Further research is needed to develop targeted pharmacological interventions to mitigate the damages of NLRP3 activation (Jo et al., 2016; Lamkanfi, 2011; Martinon et al., 2002; Petrilli et al., 2007).

Glial Cells

As stated in the previous section, microglia are the brain's resident immune cells. Microglia are charged with maintaining the brain's tissue development, homeostasis, and response to injury and repair. Microglia can adopt a wide range of phenotypes depending on the function they need to preform to maintain homeostasis (Orihuela, McPherson, &

Harry, 2016). Microglia originate around mid-embryonic development from primitive yolk sac myeloid progenitors (Alliot, Godin, & Pessac, 1999; Ginhoux et al., 2010). As a result of microglia's pivotal role in the brain, it accounts for 10-15% of the brain's total cell population (Carson, Doose, Melchior, Schmid, & Ploix, 2006).

When the brain is in a normal state (e.g., no insult or injury present), microglia maintain in their resting shape; this state resting state allows microglia to consistently monitor the brain's homeostasis (Davalos et al., 2005; Orihuela et al., 2016; Schmid et al., 2009). Microglia can induce an immune response when pathogens are present and maintain the brain's homeostasis by clearing up debris, atypical proteins, and pyroptosis (Carson et al., 2006; Fogg et al., 2006; Gautier et al., 2012; Paolicelli et al., 2011; Parwaresch & Wacker, 1984). The microglia can be stimulated into two phenotype polarizations, commonly referred to as M1 phenotype and M2 phenotype (Hu et al., 2012; Orihuela et al., 2016). Microglia M1 phenotype can be activated via ligands LPS or IFN-γ, causing the expression of proinflammatory cytokines (Olah, Biber, Vinet, & Boddeke, 2011). The microglia M2 phenotype can be initiated to commence tissue repair and combat inflammation in an anti-inflammatory capacity (Hu et al., 2012; Olah et al., 2011; Orihuela et al., 2016; Patterson, 2015).

Another glial cell type that is a part of the central nervous systems immune response are astrocytes. Within a healthy brain, astrocytes help maintain the brain's homeostasis by supporting neuronal function though sheer volume. Astrocytes make up a majority of the cells found within the brain (Abbott, Ronnback, & Hansson, 2006). Since the turn of the century, more research has been conducted into the functional role of

astrocytes in the CNS, such as, neuronal support and synaptic transmission (Sofroniew & Vinters, 2010). When an insult or injury occurs, astrocytes activate through a process called reactive astrogliosis (Sofroniew & Vinters, 2010). When reactive astrogliosis is present it indicates that damaged tissue is present, which can be caused by traumatic lesions or neurological diseases (Abbott et al., 2006; Acosta, Gioia, & Silva, 2006; Akaoka et al., 2001).

CHAPTER THREE
MATERIALS AND METHODS

Animals

Young adult male (n = 9) and female (n = 9) Sprague Dawley rats were used for this study. The rats were housed individually in a home cage with free access to food and water. $BDNF^{+/-}$ heterozygous knockout rats had a seven base pair deletion in the BDNF gene. Animals were divided into groups by sex and strain: WT females (n = 5), WT males (n = 5), BDNF KD females (n = 4), and BDNF KO males (n = 4) (SAGE Labs, Boyertown, PA). All the female animals used for this study underwent random estrous cycling that was not monitored. All procedures were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals at the University of Tennessee.

Tissue Harvest

Animals were euthanized using isoflurane overdose. Animals were decapitated and the brains were removed for dissection. Individual brains were cut at the midsagittal plane and the hippocampus from each hemisphere was dissected out. Hippocampal tissues were stored at -20°C and homogenized for protein detection via Western blot analysis.

Tissue Lysis

Hippocampal tissue was homogenized using RIPA buffer (Teknova). Tissue suspended in RIPA buffer was subjected to sonication power of 2 until the tissue was visibly solubilized in the buffer. The homogenized tissue was then allowed to rest on ice

for 1 min. The homogenate was centrifuged at 12,000 rpm for 10 minutes. Supernatant was collected in fresh tubes and used for further protein analyses.

Protein Estimation

The bicinchoninic acid method (BCA Protein Assay Kit, Thermo Scientific) was used for quantification of total protein in each sample. Protein estimation was performed using the 96-well plate method, as per the instructions of the kit. Bovine Serum Albumin (BSA) was used as the standard. Absorbance was measured at 562 nm using a Biotek plate reader and the protein concentration was calculated using the Gene5 software.

Western Blotting

The samples for western blotting were prepared by mixing 1X Laemmli buffer (BioRad) with tissue homogenate and incubating in a water bath at 100oC for 10 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by loading the samples on 4-20% precast gels (BioRad). Protein samples were then transferred on 0.45 µm nitrocellulose membrane (80V for 90 min) and blocked using 5% BSA for 1 hour at room temperature on a shaker. The blots were then incubated with primary antibody (Table 3.1) overnight at 4°C on a shaker. After the primary antibody incubation, blots were washed with 1X TBST (10 min X 3) and incubated with the respective HRP-conjugated secondary antibody. Following this, blots were washed, developed using ECL (Clarity Western ECL substrate, BioRad) and scanned using chemiluminescence on (Chemidoc Touch Imaging System, BioRad).

Table 3.1. Primary Antibodies used for Western Blotting

Primary Antibody	Catalog Number	Company
BDNF	OSB00017W	ThermoFisher Scientific
IBA-1	Ab5076	AbCam
GFAP	Ab7260	AbCam
GAP DH	Ab8245	AbCam
ASC	NBP1-78977	NovusBio
Caspase-1	NBP1-45433	NovusBio
NLRP3	NBP2-12446	NovusBio
IL-1-Beta	NB600-633	NovusBio
Beta-Actin	MAB8929	R&D Systems
Vinculin	MCA465GA	BioRad
NeuN	MAB377	Millipore
TXNIP	14715S	Cell Signaling Technologies

Quantification of Proteins

Blots were quantified using ImageJ software downloaded from NIH.gov. The band intensity was measured and corrected for the corresponding housekeeping values. Data are presented as average fold change of each group. Error bars indicate positive standard error of the mean.

Statistical Analysis

Multiple t tests were used to compare significance between sex matched groups; WT males compared to BDNF KD males; WT females compared to BDNF KD females. Statistical significance was determined using the Bonferroni-Dunn method, with alpha set to 0.05. Analyses were performed using PRISM software (version 7).

CHAPTER FOUR
RESULTS

Brain Derived Neurotrophic Factor and Heterozygous Knockout

To confirm the animals used in our experiments had the 7 base pair deletion within the BDNF gene a Western Blot was run. To verify this deletion occurred the heterozygous BDNF knock-down (BDNF KD) was compared to naïve Wild Type (WT) animals, to assess possible functional deficits. Figure 4.1 confirms that both the pro- and mature forms of BDNF were present in the hippocampal tissue collected post euthanasia for both male and female rodents. Western Blot results are shown in Figure 4.1A and 4.1B, and statistical quantification for the blots is presented in the bar graph of Figure 4.1C. The data was normalized by comparing the heterozygous BDNF knock-down (KD) ($BDNF^{+/-}$) protein expression to naïve Wild Type (WT) animals (no genetic modification). Figure 4.1 displays that the BDNF protein levels in the mutant strain are knock-down in both male and female rodent hippocampi. Verification through statistical analysis, revealed in Table 4.1A and 4.1B, yielded a reduction of pro-BDNF in males that was significant ($p < 0.001533$). However, the decrease of pro-BDNF in the KD females was not significant ($p < 0.027786$). The mature form of BDNF was confirmed through statistical analysis (Table 4.1B) and reported that there was a loss across both sexes, after the knock-down of the BDNF protein.

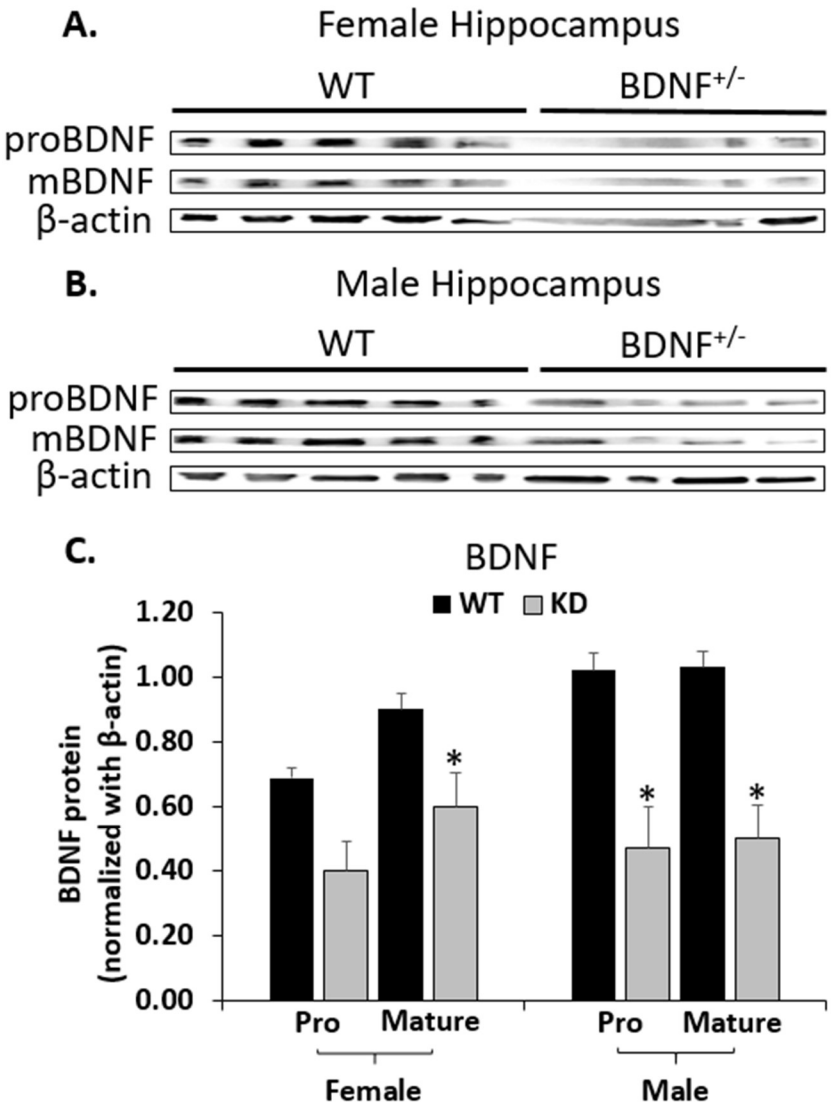

Figure 4.1. Western blot analysis confirmed BDNF heterozygosity. Tissue samples were collected from the hippocampus of adult BDNF KD and WT rats, both males and females. (A, B) Western blot images of BDNF expression in the female and male hippocampus show visual reductions in BDNF in the mutant (KD) rats, as compared to WT controls. (C) Quantification of blots indicates reductions in BDNF in both male and female KD rats. (*, $p < 0.05$). Formatting of results figure was adapted from (Mitchell, B. A., 2019).

Table 4.1. Statistical Analysis for pro-BDNF.

Gender	Significance	P-Value	Mean of WT	Mean of KD	T ratio	df	Adjusted P value*
Female	No	0.027786	0.9020	0.6025	2.768	7	0.055572
Male	Yes	0.001533	1.034	0.4950	5.019	7	0.003065
Note: * = Adjusted using Bonferroni-Dunn method.							

Table 4.2. Statistical Analysis for Mature BDNF.

Gender	Significance	P-Value	Mean of WT	Mean of KD	T ratio	df	Adjusted P value*
Female	Yes	0.013222	0.6880	0.4000	3.294	7	0.026443
Male	Yes	0.003192	1.024	0.4725	4.391	7	0.006384
Note: * = Adjusted using Bonferroni-Dunn method.							

Loss of BDNF and the Inflammasome Pathway - NLRP3

To understand the role of BDNF loss and how it affects inflammation in the central nervous system, we examined the effects of BDNF loss on Nod-like Receptor Family Pyrin Domain Containing 3 protein (NLRP3) activation and expression in the rodent hippocampus of both male and female animals. Western blot analysis for the NLRP3 protein was performed. The representative immunoblots are shown in Figure 4.2A and 4.2B and quantification of the western blots is shown in Figure 4.2C. In summary, Figure 4.2 indicates that the NLRP3 protein showed a visual increase in expression in the BDNF KD males as compared to WT males. No changes were observed in the female brain after BDNF KD. The statistical analyses are shown in table (Table 4.3).

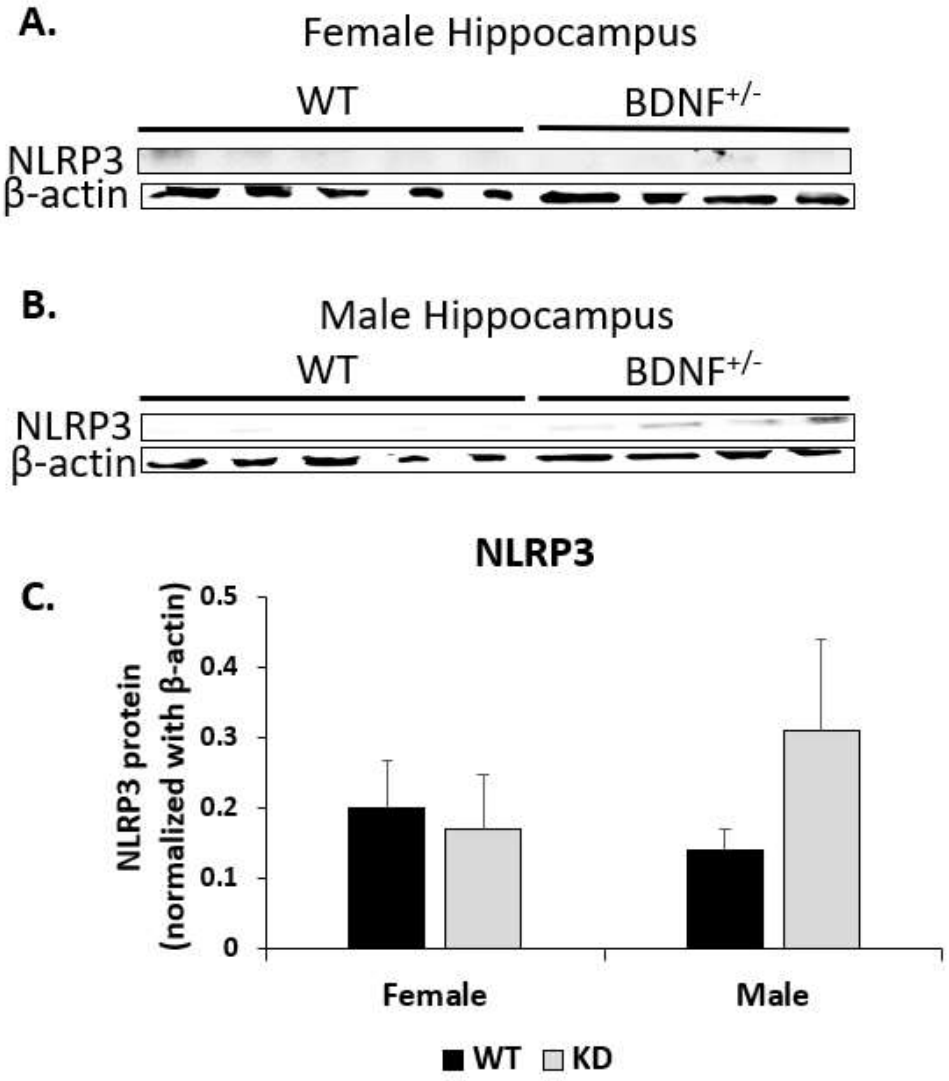

Figure 4.2. NLRP3 did not differ across sex-matched groups. The tissue samples were collected from the hippocampus of adult female and male BDNF KD and WT rats. (A) Western blot images of NLRP3 protein expression in female hippocampus samples show a slight visual decrease in the KD rats, as compared to the WT controls. (B) Western blot images of NLRP3 protein expression in male hippocampus show a visual increase in NLRP3 activation in KD rats, as compared to WT controls. (C) Quantification of blots did not indicate any significant differences between sex-matched groups. (*, $p < 0.05$).
Formatting of results figure was adapted from (Mitchell, B. A., 2019).

Table 4.3. Statistical Analysis for NLRP3.

Gender	Significance	P-Value	Mean of WT	Mean of KD	T ratio	df	Adjusted P value*
Female	No	0.433246	0.2580	0.1725	0.8313	7	0.866492
Male	No	0.195669	0.1360	0.3075	1.430	7	0.391337
Note: * = Adjusted using Bonferroni-Dunn method.							

Loss of BDNF and the Inflammasome Pathway – Apoptosis-Associated Speck-Like Protein Containing a CARD (ASC)

To further understand how NLRP3 acts due to loss of BDNF, we needed to investigate the proteins involved in the NLRP3 pathway. Western blot analysis revealed that the ASC protein was detected across both sexes in the rat hippocampus. The representative immunoblots are shown in Figure 4.3A and 4.3B. Quantification of the western blots is shown in Figure 4.3C. The statistical analyses are shown in table (Table 4.4.).

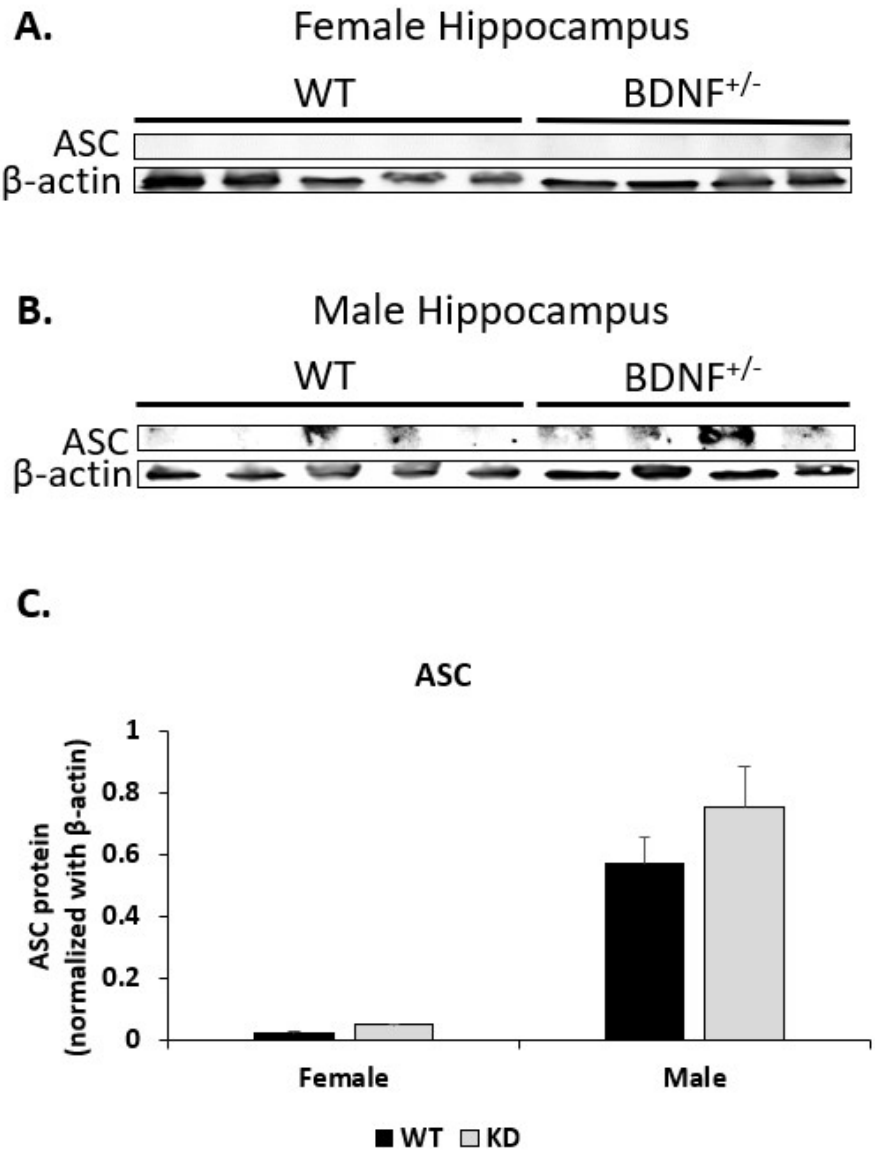

Figure 4.3. ASC did not differ across sex-matched groups. The tissue samples were collected from the hippocampus of adult female and male BDNF KD and WT rats. (A) Western blot images of ASC protein expression in female hippocampus samples are visually comparable in the KD rats when compared to the WT controls. (B) Western blot images of ASC protein expression in male hippocampus show a visual increase in ASC activation in KD rats, as compared to WT controls. (C) Quantification of blots did not indicate any significant differences between sex-matched groups. (*, $p < 0.05$).
Formatting of results figure was adapted from (Mitchell, B. A., 2019)

Table 4.4. Statistical Analysis for ASC.

Gender	Significance	P-Value	Mean of WT	Mean of KD	T ratio	df	Adjusted P value*
Female	No	0.053897	0.02600	0.06500	2.314	7	0.107794
Male	No	0.285696	0.5740	0.7500	1.156	7	0.571393
Note: * = Adjusted using Bonferroni-Dunn method.							

Loss of BDNF and the Inflammasome Pathway – Caspase 1

Caspase-1 is a protein that attaches to the NLRP3 complex and is then cleaved if the inflammasome pathway is activated. The active form of Caspase is the cleaved caspase which is an enzyme protein that catalyzes a biochemical reaction. Since we were investigating the NLRP3 pathway it was the next crucial protein to study when examining inflammasome activation. As depicted in Figure 4.4, Caspase-1 enzyme protein was detected using western blot analysis and the representative immunoblots are shown in Figure 4.4 A and 4.4 B. Quantification of the western blots is shown in Figure 4.4 C. In summary, Figure 4.4 indicates that the enzyme protein levels of Caspase-1 in the female rodent hippocampus show a decrease in Caspase-1 activation from the BDNF KD to the WT, however, quantification revealed this decrease is not statistically significant Table (4.5.). In the male rodent hippocampus, there was a significant increase in Caspase-1 activation in the BDNF KD when compared to the WT. This further confirms in relation to the above results that the inflammasome pathway is being activated primarily in the male brain following BDNF loss.

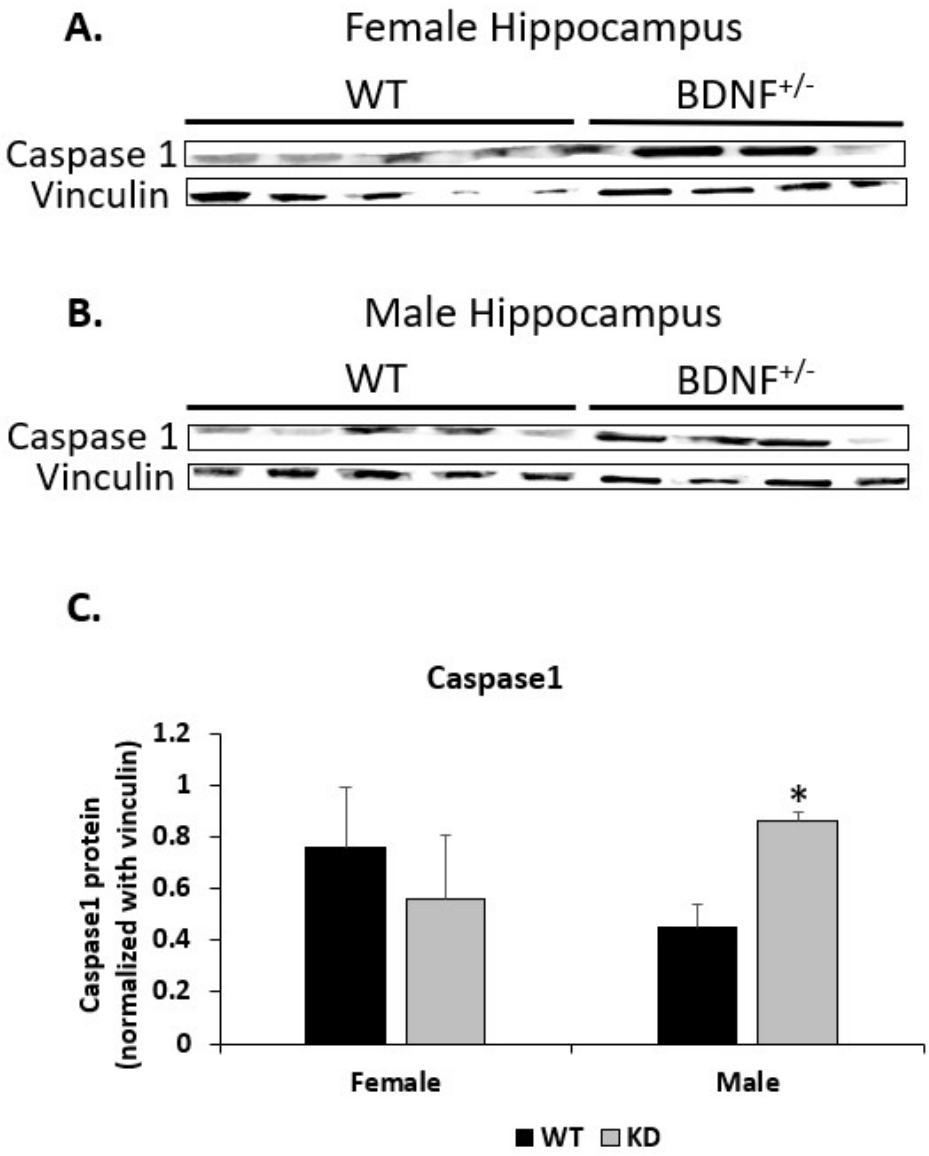

Figure 4.4. Caspase1 activation was increased in BDNF KD males. Tissue samples were collected from the hippocampus of adult BDNF KD and WT rats, both males and females. (A) Western blot images of Caspase1 protein expression in female hippocampus show a decrease of Caspase-1 activation from the WT controls to the BDNF KD rats. (B) Western blot images of Caspase1 protein expression in male hippocampus show a visual increase in the KD rats, as compared to WT controls. (C) Quantification of immunoblots showed a significant increase in Caspase1 protein in the knock-down males. The difference between female groups is not statistically significant. (*, $p < 0.05$).

This formatting of results figure was adapted from (Mitchell, B. A., 2019)

Table 4.5. Statistical Analysis for Caspase-1.

Gender	Significance	P-Value	Mean of WT	Mean of KD	T ratio	df	Adjusted P value*
Female	No	0.579414	0.7620	0.5650	0.5811	7	>0.999999
Male	Yes	0.012790	0.4480	0.8600	3.502	6	0.025580
Note: * = Adjusted using Bonferroni-Dunn method.							

Loss of BDNF and the Inflammasome Pathway – Interleukin-1 Beta (IL1-β)

Interleukin-1 beta (IL1-β) is an active proinflammatory cytokine that once activated is released outside of the cells to interact with its receptors and activate the complex inflammatory cascade. We examined the effects of BDNF KD on IL1-βactivation in the rodent hippocampus of both females and males. As depicted in Figure 4.5 IL1-β protein was detected using western blot analysis and the representative immunoblots are shown in Figure 4.5 A and 4.5 B. Quantification of the western blots is shown in Figure 4.5 C. The statistical analyses are shown in table (Table 4.6.).

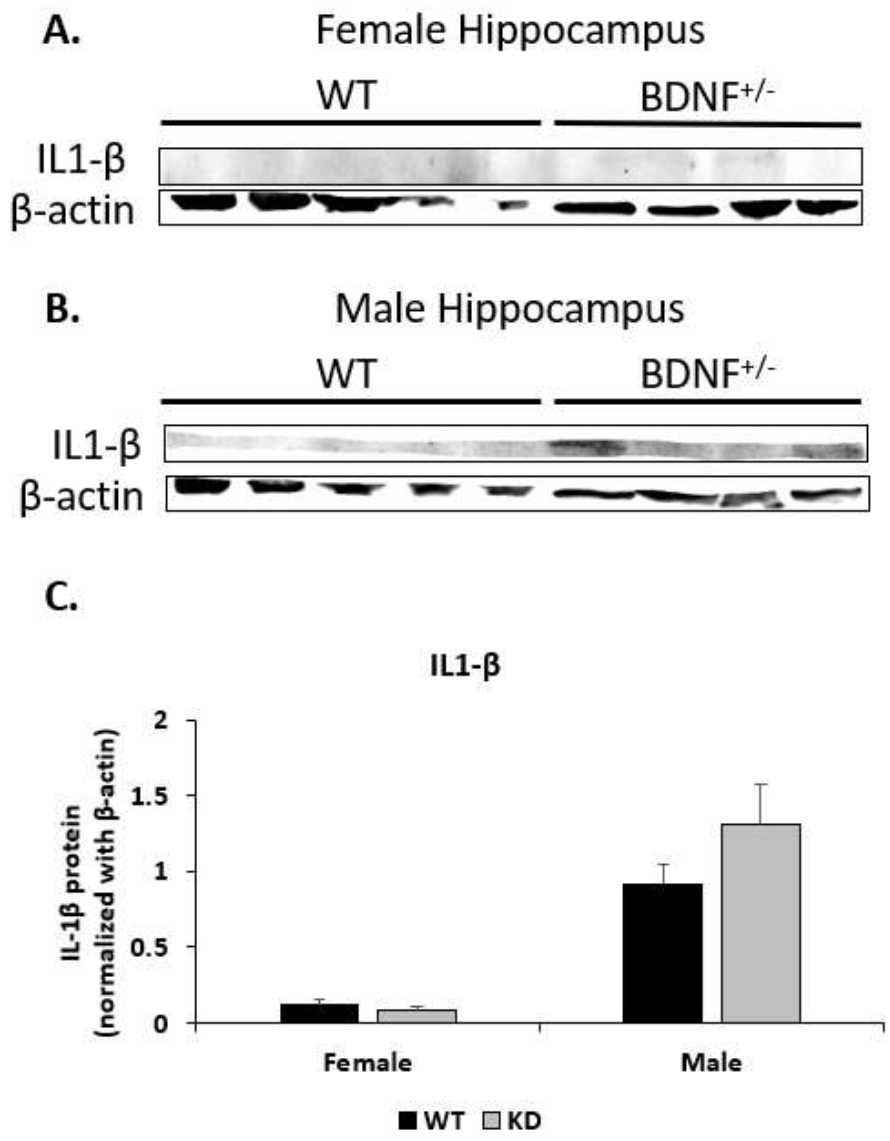

Figure 4.5. IL1-Beta did not differ across sex-matched groups. The tissue samples were collected from the hippocampus of adult female and male BDNF KD and WT rats. (A) Western blot images of IL1-Beta protein expression in female hippocampus samples are visually comparable in the KD rats when compared to the WT controls. (B) Western blot images of IL1-Beta protein expression in male hippocampus show are visually comparable in IL1-Beta activation in KD rats, as compared to WT controls. (C) Quantification of blots did not indicate any significant differences between sex-matched groups. (*, $p < 0.05$).
Formatting of results figure was adapted from (Mitchell, B. A., 2019)

Table 4.6. Statistical Analysis for IL1-Beta.

Gender	Significance	P-Value	Mean of WT	Mean of KD	T ratio	df	Adjusted P value*
Female	No	0.423692	0.1180	0.07750	0.8495	7	0.847384
Male	No	0.194904	0.9140	1.310	1.433	7	0.389808
Note: * = Adjusted using Bonferroni-Dunn method.							

Thioredoxin-Interacting Protein (TXNIP)

Thioredoxin-interacting protein (TXNIP) is a marker known to act as an oxidative stress mediator by suppressing thioredoxin activity. We examined the effects of BDNF KD on TXNIP protein expression in male and female rodents. As seen in Figure 4.6 the TXNIP protein expression was detected using western blot analysis and representative immunoblots are shown in Figure 4.6 A and 4.6B. Quantification of the western blots is shown in Figure 4.6C. In summary, Figure 4.6 indicates that the levels of TXNIP protein visually shown a slight decrease in the BDNF KD females when compared to the WT females, however, quantification revealed that the difference is not statistically significant. In the males a visual increase is seen for TXNIP activation and when quantified revealed that there is a significant increase in TXNIP activation in BDNF KD males when compared to WT rats. These results suggest that BDNF loss leads to increased oxidative stress in the male hippocampus but not the female. The statistical analyses are shown in table (Table 4.7).

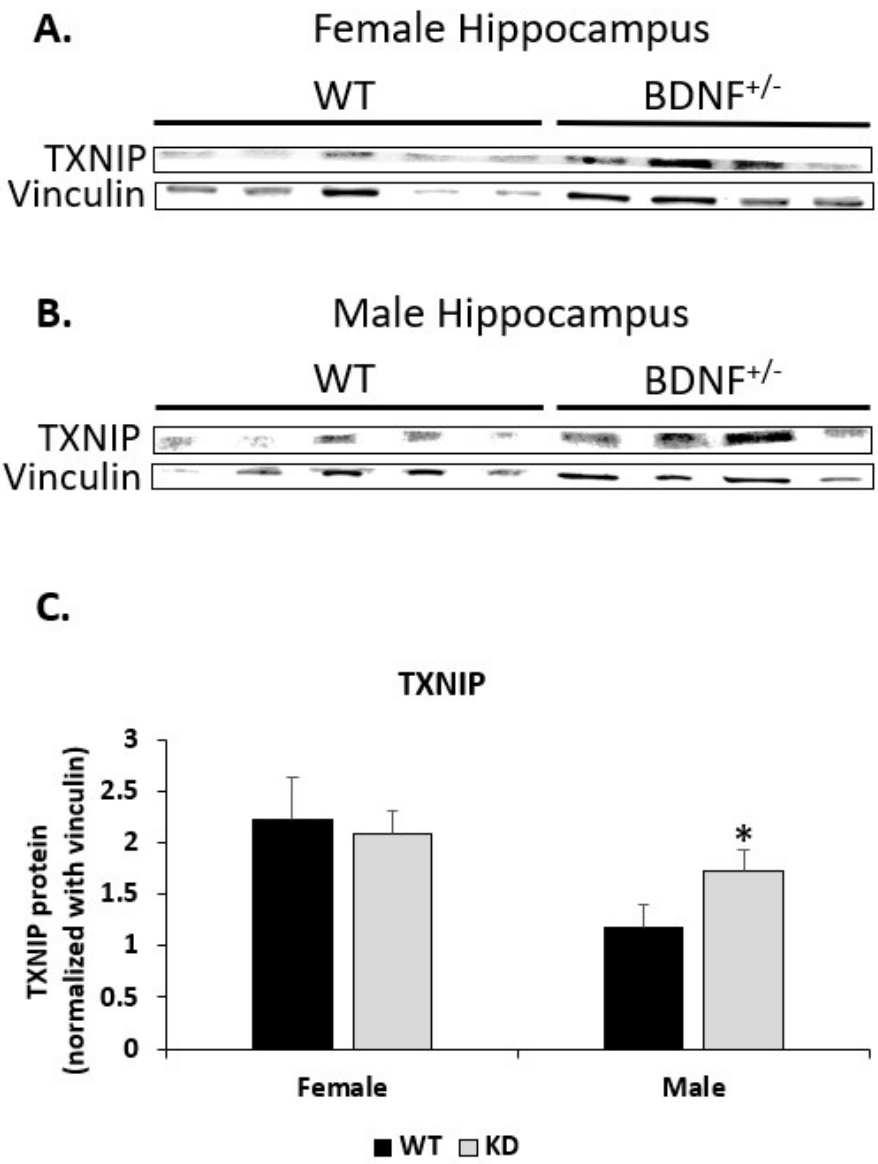

Figure 4.6. TXNIP was increased in BDNF KD males. Tissue samples were collected from the hippocampus of adult BDNF KD and WT rats, both males and females. (A) Western blot images of TXNIP protein expression in female hippocampus samples are comparable to the KD rats, as compared to WT controls. (B) Western blot images of TXNIP protein expression in male hippocampus show a visual increase in the KD rats, as compared to WT controls. (C) Quantification of immunoblots showed a significant increase in TXNIP protein in the knock-down males. The difference between female groups is not statistically significant. (*, $p < 0.05$).

This formatting of results figure was adapted from (Mitchell, B. A., 2019)

Table 4.7. Statistical Analysis for TXNIP.

Gender	Significance	P-Value	Mean of WT	Mean of KD	T ratio	df	Adjusted P value*
Female	No	0.778769	2.223	2.095	0.2965	5	>0.999999
Male	Yes	0.011208	0.9725	1.733	3.612	6	0.022416
Note: * = Adjusted using Bonferroni-Dunn method.							

Ionized Calcium Binding Adaptor Molecule1 (IBA1)

Ionized calcium binding adaptor molecule 1 (Iba1) is a protein marker that is specific for microglia/macrophages calcium-binding protein. This marker is widely used to indicate immune system activity. We tested the effects of BDNF KD on Iba1 activation in the hippocampus of both male and female rodents. As depicted in Figure 4.7 IL1-Beta protein was detected using western blot analysis and the representative immunoblots are shown in Figure 4.7 A and 4.7 B. Quantification of the western blots is shown in Figure 4.7 C. In summary, Figure 4.7 indicates that no significant changes were observed in microglia activation of male brains. Iba1 was decreased in BDNF KD females, this suggests that a full-blown inflammatory cascade is not triggered at basal level after BDNF loss, but the male brain remains primed for inflammation via activation of the NLRP3 inflammasome pathway, even despite BDNF knockdown. The statistical analyses are shown in table (Table 4.8.)

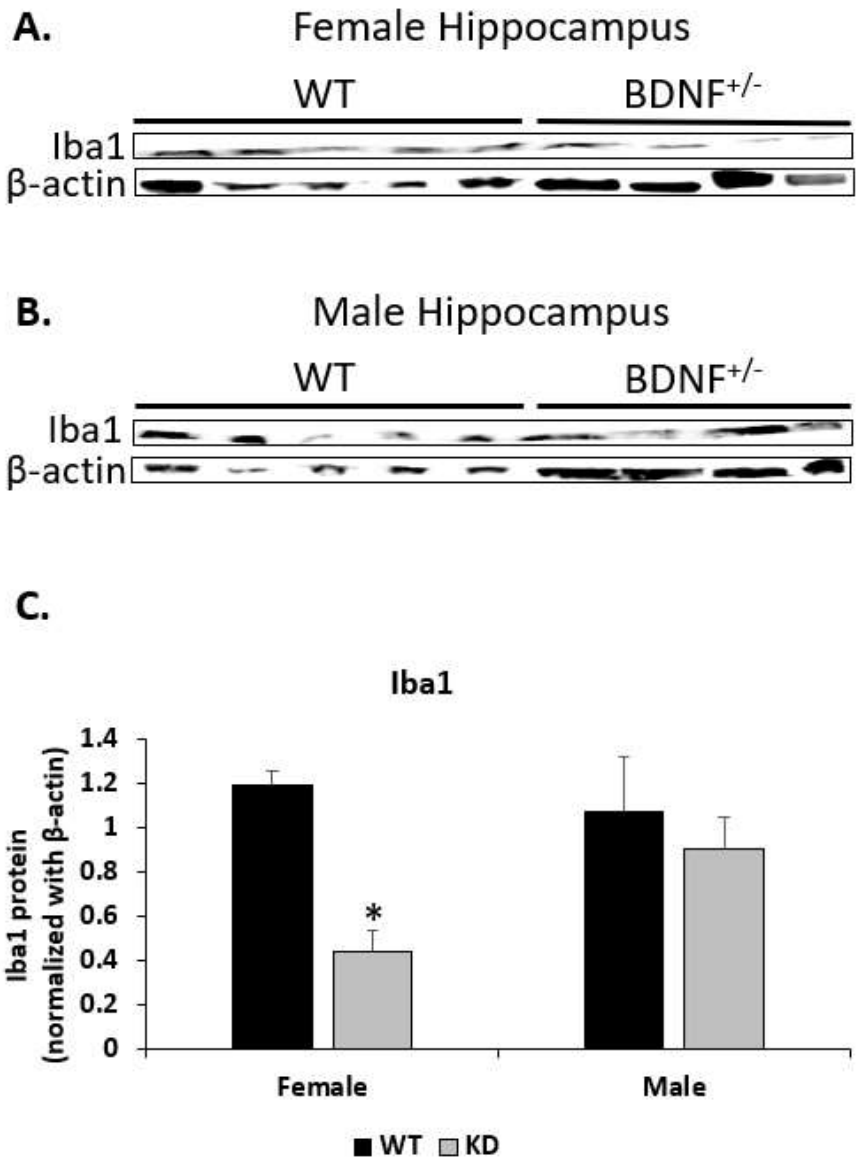

Figure 4. 7. Iba1 was decreased in BDNF KD females. Tissue samples were collected from the hippocampus of adult BDNF KD and WT rats, both males and females. (A) Western blot images of Iba1 protein expression in female hippocampus samples show a visual decrease comparable to the KD rats, as compared to WT controls. (B) Western blot images of Iba1 protein expression in male hippocampus show a visual increase in the KD rats, as compared to WT controls. (C) Quantification of immunoblots showed a significant increase in Iba1 protein in the knock-down females. The difference between male groups is not statistically significant. (*, $p < 0.05$). Formatting of results figure was adapted from (Mitchell, B. A., 2019)

Table 4.8. Statistical Analysis for Iba1.

0Gender	Significance	P-Value	Mean of WT	Mean of KD	T ratio	df	Adjusted P value*
Female	Yes	0.000449	1.193	0.4350	6.925	6	0.000898
Male	No	0.581003	1.070	0.9025	0.5832	6	>0.999999
Note: * = Adjusted using Bonferroni-Dunn method.							

Glial Fibrillary Acidic Protein (GFAP)

Glial fibrillary acidic protein (GFAP), is a protein marker that is expressed by astrocytes in the central nervous system (CNS). We looked for changes in GFAP expression in the hippocampus of both male and female rodents. As depicted in Figure 4.7 GFAP protein was detected using western blot analysis and the representative immunoblots are shown in Figure 4.7 A and 4.7 B. Quantification of the western blots is shown in Figure 4.7 C. In summary, Figure 4.7 indicates that no significant changes were observed in astrocyte activation for either male or female brains. The statistical analyses are shown in table (Table 4.9.).

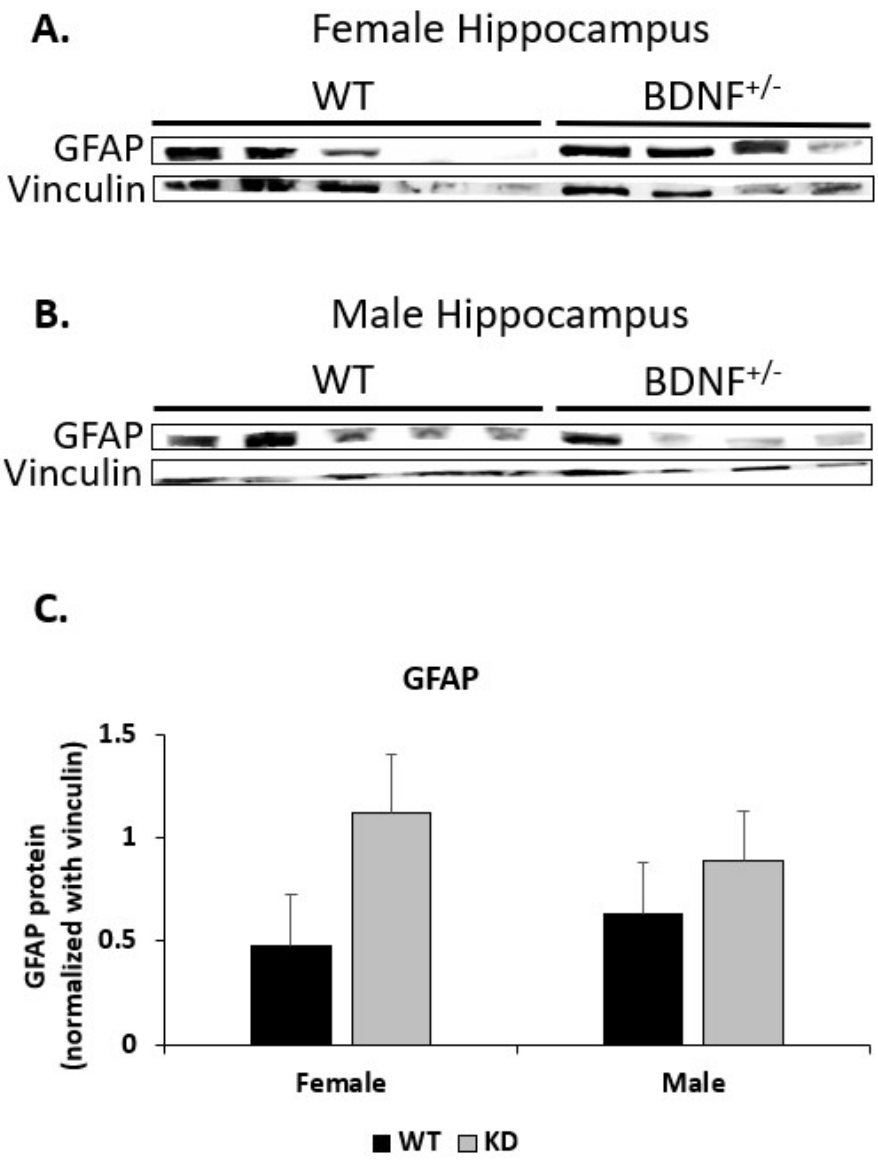

Figure 4.7. GFAP did not differ across sex-matched groups. The tissue samples were collected from the hippocampus of adult female and male BDNF KD and WT rats. (A) Western blot images of GFAP protein expression in female hippocampus samples are visually comparable in the KD rats when compared to the WT controls. (B) Western blot images of GFAP protein expression in male hippocampus show are visually comparable in GFAP activation in KD rats, as compared to WT controls. (C) Quantification of blots did not indicate any significant differences between sex-matched groups. (*, $p < 0.05$). Formatting of results figure was adapted from (Mitchell, B. A., 2019)

Table 4.9 Statistical Analysis of GFAP

Gender	Significance	P-Value	Mean of WT	Mean of KD	T ratio	df	Adjusted P value*
Female	No	0.114220	0.4820	1.123	1.804	7	0.228439
Male	No	0.548744	0.6340	0.8875	0.6299	7	>0.999999
Note: * = Adjusted using Bonferroni-Dunn method.							

CHAPTER FIVE
DISCUSSION AND CONCLUSIONS

Discussion

As mentioned previously, we hypothesize that BDNF heterozygous rats will have altered protein levels of inflammatory markers due to reduction of BDNF expression, and that this may occur in a sex specific manner. We tested this hypothesis through the examination of inflammation within the Central Nervous System (CNS), specifically through examining the NLRP3 inflammasome pathway. We verified this hypothesis by using sex matched male and female groups comprised of $BDNF^{+/-}$ KD and WT rats.

Our first aim was to understand the basal relationship between BDNF and how its neuronal functioning affects neuroinflammatory pathways. In accordance with our hypothesis our study conveys that: BDNF loss changed the protein expression levels of NLRP3, ASC, Caspase-1, and IL1-β. Our second aim was to determine if sex-differences play a role in BDNF regulation and neuroinflammation. Our findings indicated that all four of these inflammasome markers showed a pattern of increase only in the male hippocampi (HPC) and not in the females after BDNF KD. These results lead us to believe that the male brain primes for inflammation under reduced levels of BDNF.

Additionally, we found an increase in oxidative stress (TXNIP) was observed in males but not in female rats after BDNF loss. These results indicate that increased inflammation after BDNF reduction could be mediated via increased oxidative stress. Protein expression studies of microglia specific marker, Iba1, and astrocyte specific marker, GFAP, showed no significant changes after BDNF loss. These results compel us

to believe that BDNF reduction leads to priming of the inflammation in the brain and not a full-blown inflammatory state. This is observed in a sex specific manner, with effects seen only in the male HPC. We further hypothesize that due to a pre-existing BDNF loss and primed inflammation, the brain will have a worsened outcome after any neuronal insult. Parallel studies in our lab have found that estrogen in reproductive-aged females has neuroprotective effects post-BDNF loss (Mitchell, B. A., 2019), which was corroborated in our study (i.e., males had priming of inflammation over females).Future endeavors should target more effective treatment options for neurological and psychiatric diseases using BDNF as an early detection biomarker for neuroinflammation.

Limitations of the Study

Future directions for this research to have a higher impact would be to increase the sample size to increase statistical power. Further studies are needed to confirm that males are the only sex experiencing the priming of the NLRP3 inflammasome pathway. Additional parallel studies could investigate the effects of BDNF loss in other limbic structures: striatum, cortex, thalamus, and brainstem. This investigation has deduced that BDNF loss primes the brain to inflammasome activation, and likely more so in male animals. Consequently, we suggest that further investigation is needed on the pattern and timing of inflammation activation resulting in sex dependent changes of neurotrophin expression. We must acknowledge that sex dependent factors are key components of neurophysiology and will vary accordingly. The trends witnessed within our study could be explained by the priming of inflammation due to oxidative stress and in males because of the absence of E2 within the CNS (Thakkar, Wang, Wang, Vadlamudi, & Brann, 2018).

However, this investigation is limited to the HPC. Future objectives for this study will include analysis of additional limbic structures, inflammasome, and glial cell protein markers. Additional studies on disease models and aged animals may be beneficial.

Conclusions

This study provides meaningful information for pre-clinical animal models, with crucial translational value. We have concluded that when BDNF is reduced, a cascade of neurotrophic downstream signaling is inhibited or disrupted. BDNF in both its pro and mature forms is essential for healthy brain function. This investigation has illustrated that BDNF loss activates the NLRP3 inflammasome pathway, setting forth a cascade of inflammation activation, increasing oxidative stress and culminating in an immune response from glial cells in the CNS. We also highlighted that the male and female brain handle BDNF loss differently: reproductive aged females do not experience the same priming for inflammation that males experience. This study was novel because BDNF has not been extensively assessed in healthy non-disease model brains, not have sex differences have been examined. We believe this information will aid researchers in their future endeavors to create targeted treatment therapies for neurodegenerative and psychiatric diseases.

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VITA

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